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13. ABSTRACT (Maximum 200 Words)

Endothelial cells must express plasminogen activator inhibitor type-1 (PAI-1) in order to undergo and angiogenic switch during tumorigenesis. The PAI-1 gene has emerged, therefore, as an important candidate target for gene therapy of human breast cancer. In year 03, we conducted studies to confirm that targeted ablation of endothelial cell PAI-1 gene expression resulted in a marked inability to migrate and an inability to from angiogenic networks on Matrigel. Addition of recombinant active PAI-1 restored migratory of these genetically-engineered PAI-1-deficient cells to approximate that of wild-type endothelial cells. Confirmatory results were obtained with human microvessel endothelial (HMEC-1) cells which validates these findings within the context of human endothelial cells and human endothelial tubulogenic differentiation. Capillary network integrity (i.e., angiogenic structures for by T2 and HMEC-1 cells) on Matigel surfaces was disrupted by addition of neutralizing PAI-1 antibodies. This suggests that continued PAI-1 synthesis and/or activity, even in mature tubes, was required for network stability. These results support the "balanced proteolysis" concept of angiogenesis and support our hypothesis that the PAI-1 gene is a anti-angiogenic target for breast cancer therapy.

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INTRODUCTION

Continued growth of a malignant tumor beyond a certain critical size is dependent on the development of a network of feeder blood vessels (1,2). Findings from a number of laboratories have indicated that this "angiogenic switch" is highly-dependent on the temporally-regulated and focalized localization of several extracellular proteases and protease inhibitors involving members of both the plasmin-based and metalloproteinase cascades (3,4). *In vitro* analysis of the requirements for the formation of endothelial tubular networks in various culture model systems implicated both urokinase plasminogen activator (uPA) and its fast-acting type-1 inhibitor (PAI-1) as necessary to achieve complete angiogenesis, consistent with the "balanced proteolysis" concept of endothelial cell migration (5-7). Recent data in mice genetically-engineered to be deficient in expression of genes that encode specific elements of the plasmin activation system has confirmed the critical importance of PAI-1 synthesis in tumor-induced angiogenesis (5-7). Indeed, the absence of host PAI-1 completely inhibited local invasion and vascularization of transplanted malignant tumors in PAI-1 null mice (5,6). This inability to mount an angiogenic response, moreover, prevented invasive growth by an aggressive and metastatic tumor type (5,6).

Breast tumors with high PAI-1 levels, in particular, are fast-growing carcinomas with a well-developed angiogenic network, a high incidence of metastatic spread, early recurrence and poor prognosis (8, reviewed in 9). PAI-1 as a determinant in aggressive growth behavior is particularly important in the progression of mammary carcinoma. Most recently, PAI-1 has been shown to be markedly promigratory for invasive breast cancer cells, an effect attenuated by direct inhibition of PAI-1 function (10). These data highlight the potential relevance of PAI-1 modulation as a means to treat human breast cancer and complement our own work on genetic targeting of PAI-1 mRNA transcripts with subsequent attenuation of cellular motile traits (11-13).

The goals of this investigation are to determine the level of endothelial cell PAI-1 expression necessary for development and maintenance of the breast tumor-induced angiogenic phenotype in a 3-D culture model of breast tumor-stromal-endothelial cell interactions that mimics the *in vivo* disease state. Gene therapy approaches using antisense vector constructs as well as homologous recombination methods previously developed in this laboratory are utilized to directly disrupt PAI-1 gene expression in cultured endothelial cells. The consequences of this targeted disruption on the ability of endothelial cells to form branching angiogenic networks in response to co-culture with human breast cancer cells will be evaluated. This study constitutes the first comprehensive assessment of PAI-1 genetic therapy as an approach to inhibit growth of human breast cancers by targeting a gene essential for the angiogenic process.

BODY

Studies in year 03 of this investigation were directed to the goals described in Task 2 of the approved statement of work.

Task 2. To determine the consequences of molecular genetic down-regulation of endogenous

PAI-1 gene expression on the ability of wild-type T2 endothelial (T2-WT) cells to form branching angiogenic networks upon co-culture with human breast carcinoma cells and to migrate through matrix barriers in response to tumor-derived stimuli. This is necessary to assess the extent to which endogenous PAI-1 gene expression is susceptible to genetic manipulation and the window of down-regulation required to achieve a defined and quantifiable therapeutic result (i.e., a reduction in, or loss of, the angiogenic response).

We created PAI-1 functional "knockout" endothelial cells using T2 cells as the parental strain by transfection of a PAI-1 antisense expression vector (10,11). While T2 cells formed tubular networks when placed in culture over a Matrigel substrate and expressed high levels of PAI-1 mRNA, antisense vector-transfectants did not exhibit an angiogenic response (**Figure 1**). T2 wild-type endothelial cells did exhibit an angiogenic switch when plated on Matrigel-coated surfaces. A robust angiogenic response (complete with gel invasion, sprout formation from the lateral surface of the tubular structures, complex branch patterns in 3-D orientation), however, required co-culture with MDA-MB-231 human breast carcinoma monolayers. Northern blot analysis confirmed that the T2/IAP antisense cell line did not express PAI-1 transcripts (**Figure 1**) and that the down-regulation of expression achieved was, at the protein level, specific for PAI-1 (11). A similar approach was used to create the PAI-1 functionally-null cell line 4HH (12). While wild-type T2 cells were capable of forming extensively branched capillary networks in a complex 3-D gel consisting of a 3:1 mixture of Vitrogen-Matrigel, 4HH cells were incapable of lattice formation and effectively degraded the gel scaffold **(Figure 2)**.

Since these data provided proof of principle (i.e., that inhibition of PAI-1 ablated *in vitro* angiogenesis), we derived a stable PAI-1-null T2 cell line by molecularly-disrupting the endogenous gene with a targeting vector. The resulting cell line (T2-null) similarly failed to form branched angiogenic networks *in vitro*. T2-null cells were subsequently transfected with the PAI-1 sense expression vector Rc/CMVPAI (8,9) (**Figure 1**) and four neomycin (G418)-resistant cell lines were derived that varied in the level of vector-driven PAI-1 transcript expression and migratory ability (**Table 1**). Unlike T2 cells (or the related 4HH cell line) where PAI-1 expression is ablated by antisense PAI-1 (9), antisense c-*fos* (10) constructs or by targeted gene disruption and which are poorly motile and non-angiogenic (**Table 1**), vector-mediated PAI-1 "rescue" restored (to varying extents) cellular motile ability.

Table 1. Effect of PAI-1 expression targeting and vector "rescue" on cell motility using a quantifiable assay of planar locomotion[a]

Cell Line	Method of PAI-1 Expression Disruption	Relative Motility[b]
T2	None (wild-type)	100
T2/IAP	Rc/CMVIAP transfection	40 ± 8
T2-null	Targeted disruption vector	37 ± 5
T2-nullR1	Disruption vector⁄Rc/CMVPAI	56 ± 4
T2-nullR2	Disruption vector⁄Rc/CMVPAI	78 ± 9
T2-nullR3	Disruption vector⁄Rc/CMVPAI	93 ± 7
T2-nullR4	Disruption vector⁄Rc/CMVPAI	49 ± 3

[a] Wounds were created by pushing the narrow end of a sterile P1000 plastic pipette tip (Continental Laboratory Products, San Diego, CA) through the monolayer. Cultures were incubated in the existing media for times indicated in the text. Wound closure was assessed by time-lapse photomicroscopy and injury repair rates calculated, as a function of time, from measurements made utilizing an inverted microscope fitted with a calibrated ocular grid.

[b] Relative motility = distance migrated in 24 hours compared to wild-type T2 cells.

The data presented in **Figures 1** and **2** as well as in **Table 1** implicate PAI-1 as an important element in the cellular motile process. To further evaluate the role of PAI-1 in various endothelial cell functions (i.e., matrix adhesion, motility, tubulogenesis), two endothelial cell lines (T2-WT, HMEC-1) were used in experiments to: (a) specifically attenuated PAI-1 synthesis by by transfection with the Rc/CMVIAP vector (as above), followed by attempts to "rescue" endothelial function by addition of exogenous active PAI-1 protein, or (b) inhibit PAI-1 activity with a neutralizing antibody. Genetically-targeted PAI-1 down-regulation with the antisense approach effectively inhibited T2 cell migration **(as in Table 1)** and this motile deficit could be rescued by addition of active PAI-1 protein; a similar rescue was evident in the PAI-1 functionally-null 4HH cell line **(Figure 3)**. Use of PAI-1 neutralizing antibodies similarly attenuated T2 cell locomotion and also promoted substrate detachment of both T2 and HMEC-1 cells. PAI-1 activity was required for endothelial tubulogenesis on Matrigel surfaces as addition of neutralizing antibodies blocked cellular coalescence into tubular networks. This effect is consistent with the block in tubulogenesis attained by Rc/CMVIAP transfection **(Figures 1 and 2)** but, more specifically, implicate PAI-1 activity as required for tube formation. Importantly, the integrity of capillary networks formed by both WT-T2 and HMEC-1 endothelial cells was disrupted by addition of neutralizing PAI-1 antibodies suggesting that continued PAI-1 synthesis, even in "mature" tubes, was required for network stability. Collectively, these data indicate that PAI-1 expression is an essential and targetable aspect of a successful angiogenic response in vitro. The results presented in this annual report, therefore, support our hypothesis tht therapies directed at perturbation of PAI-1 synthesis or function in developing as well as mature angiogenic network structures may have significant benefit in the management of human brerast tumors.

KEY RESEARCH ACCOMPLISHMENTS

The key accomplishments achieved during the report period are as follows:

1. Confirmed that targeted ablation of endothelial cell PAI-1 gene expression, using antisense expression vectors (Rc/CMVIAP) resulted in marked inhibition of cell motility and an inability to form angiogenic networks on Matrigel-coated surfaces.

2. Addition of recombinant PAI-1 to cultures of T2 endothelial cells with genetically-attenuated PAI-1 levels restored their migratory rate to approximate that of wild-type cells. Similar positive effects on the migration of the PAI-1 functionally-null 4HH cell line was achieved by exogenous PAI-1 supplementation. This approach to motility assessments confirmed that PAI-1 is a critical element in the cellular migratory program.

These finding support the likelihood that the overall experimental strategy to target PAI-1 expression in tumor angiogenesis will result in the design of genetic approaches that will have a defined therapeutic applicability.

3. Confirmatory results were obtained in the human microvessel endothelial cell line HMEC-1 which validates findings in the well-characterized T2 and 4HH cell systems in the context of human endothelial cells and tubulogenic differentiation.

4. Importantly, the integrity of capillary networks formed by both WT-T2 and HMEC-1 endothelial cells was disrupted by addition of neutralizing PAI-1 antibodies suggesting that continued PAI-1 synthesis, even in "mature" tubes, was required for network stability. Collectively, these data indicate that PAI-1 expression is an essential and targetable aspect of a successful angiogenic response in vitro.

REPORTABLE OUTCOMES

1. The following manuscripts have been published or are in press and cite support from grant DAMD17-00-1-0124.

Kutz, S.M., Hordines, J., McKeown-Longo, P.J., and Higgins, P.J. (2001) TGF-β1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. Journal of Cell Science 114:3905-3914.

Providence, K.M., Staiano-Coico, L., and Higgins, P.J. (2001) A quantifiable *in vitro* model to assess the effects of PAI-1 gene targeting on epithelial cell motility. In: ;Wound Healing: Methods and Protocols (DiPietra, L., Editor), Humana Press.

Samarakoon, R. and Higgins, P.J. (2002) MEK/ERK pathway mediates cell-shape-dependent plasminogen activator inhibitor type-1 gene expression upon drug-induced disruption of the microfilament and microtubule networks. Journal of Cell Science 115, 3093-3103.

Providence, K.M., White, L.A., Tang, J., Gonclaves, J., Staiano-Coico, L., and Higgins, P.J. (2002). Epithelial monolayer wounding stimulates binding of USF-1 to an E box motif in the plasminogen activator inhibitor type-1 gene. Journal of Cell Science 115, 3767-3777.

Samarakoon, R. and Higgins, P.J. (2002) $pp60^{c\text{-}src}$ integrates cytoskeletal dynamics, MAP kinase activation and PAI-1 gene regulation. In: Recent Research Developments in Biochemistry 3, 157-167.

Samarakoon, R. and Higgins, P.J. (2003) $pp60^{c\text{-}src}$ mediates ERK activation/nuclear localization and PAI-1 gene expression in response to cellular deformation. Journal of Cellular Physiology 195, 411-420.

Providence, K.P and Higgins, P.J. (2003) PAI-1 gene expression is required in two distinct phases of induced epithelial cell migration. Journal of Cellular Physiology (in press).

Qi, L. and Higgins, P.J. (2003) Use of chromatin immunoprecipitation to identify E box-binding transcription factors in the promoter of the growth state-regulated human PAI-1 gene. Recent Research Developments in Molecular Biology (in press).

2. A new Idea proposal entitled "Inducible Anti-Angiogenic Gene Therapy" was recently funded by the DOD Breast Cancer Program.

3. Vectors have been created (Rc/CMVPAI, Rc/CMVIAP) that can be distributed to DOD investigators involved in angiogenesis research.

CONCLUSIONS

Several important conclusions were derived as a result of work initiated and completed during the period covered by this report.

- PAI-1 expression is required for optimal endothelial cell migration *in vitro*.
- Endothelial cell motile deficits, produced as a consequence of PAI-1 expression targeting, can be restored to approximately wild-type levels by addition of exogenous "active" PAI-1 protein.
- Confirmatory results were obtained in the human microvessel endothelial cell line HMEC-1 which validates findings in the well-characterized T2 and 4HH cell systems in the context of human endothelial cells and tubulogenic differentiation.
- The integrity of capillary networks formed by both WT-T2 and HMEC-1 endothelial cells was disrupted by addition of neutralizing PAI-1 antibodies suggesting that continued PAI-1 synthesis, even in "mature" tubes, was required for network stability.
- These data are inconsistent with the emerging realization that "balanced proteolysis", in general, is an essential aspect of a successful angiogenic response and that PAI-1, in particular, is a major regulator of tumor-dependent angiogenesis.
- It is possible to design targeted genetic therapies to manipulate expression of an important pro-angiogenic gene (PAI-1) under defined *in vitro* conditions.

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APPENDIX

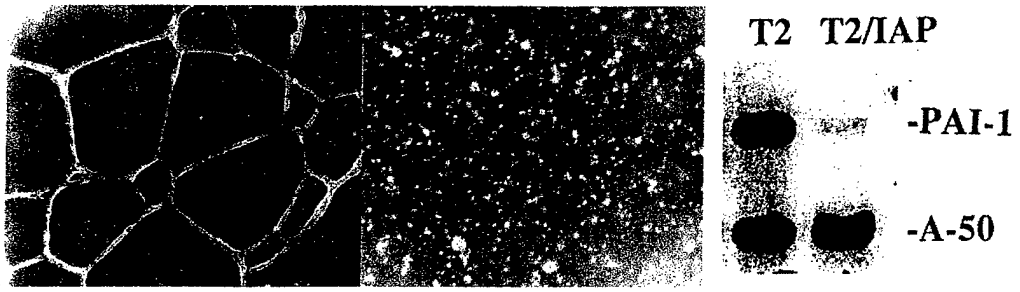

Figure 1. Capillary formation on Matrigel requires migration to achieve tubular coalescence and provides an easily accessible culture system to deliver plasmids, antibodies, reagents to a differentiated capillary network. Plating of T2 cells onto Matrigel results in the formation of tubular networks with luminal spaces (left panel). Northern analysis of harvested tubular structures indicated that cells in these networks expressed abundant PAI-1 transcripts (right panel). In contrast, T2 cells stably-transfected with the Rc/CMVIAP antisense construct expressed relatively low levels of PAI-1 mRNA (right panel) and failed to migrate and coalesce into tubular structures (middle panel). T2/IAP cells remained as single cells (without any evidence of a migratory track) or formed small multicellular aggregates in Matrigel. PAI-1 expression , thus, is required for tubular differentiation in Matrigel.

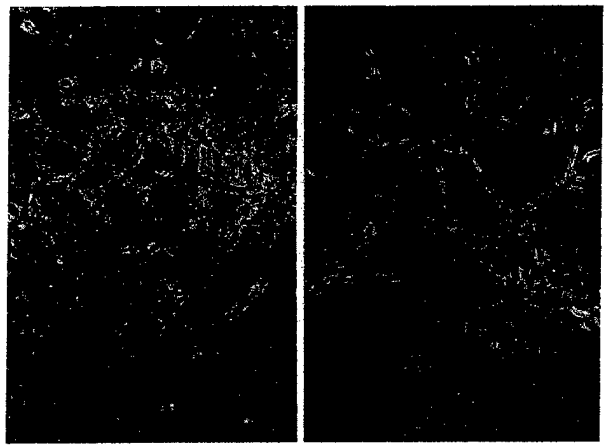

Figure 2. Visual analysis of the ability of the PAI-$1^{-/-}$ 4HH (left panel) and wild-type T2 (right panel) cells to form capillary networks in a complex "in gel" support matrix consisting of a 3:1 mixture of Vitrogen-Matrigel. Cultures were photographed 7 days after initiation of suspension culture. 4HH cells failed to form any real network structure and extensively degraded the gel matrix. T2 cells, in comparison, constructed highly branched and anastomizing capillary networks. Many of these tubular processes had lumen-like structures. Extensive sprout formation was evident at the tips of T2 branches indicating that T2 cell in-gel cultures formed both invasive and differentiated compartments.

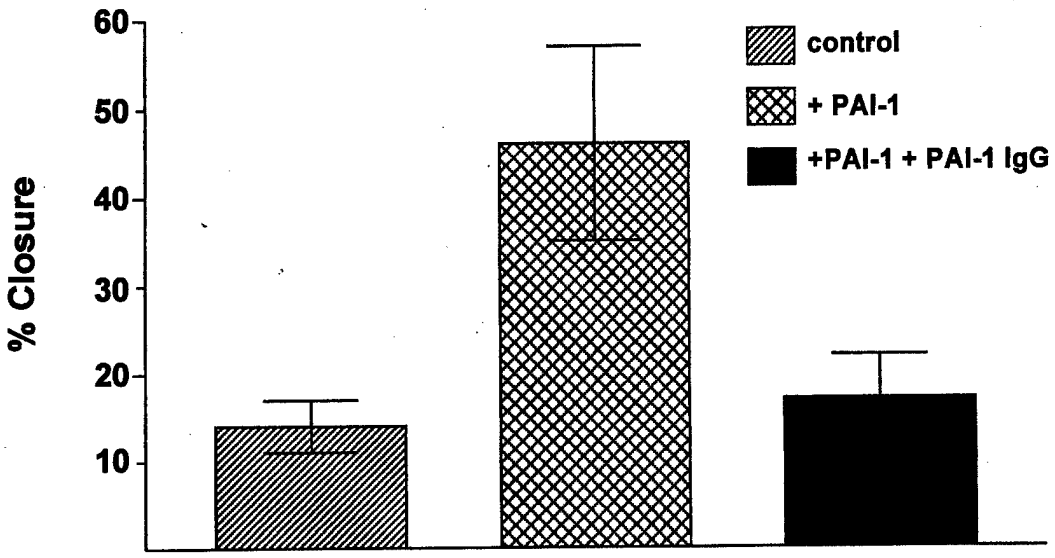

Figure 3. Stimulation of cell migration by exogenous addition of PAI-1 to scratch-injured 4HH cell monolayers. Wound closure in 4HH cultures increased approximately 4-fold following addition of active recombinant PAI-1 (1 μg/ml). PAI-1-augmented cell motility was effectively ablated by co-incubation of PAI-1 with neutralizing PAI-1 antibodies prior to addition to wounded 4HH monolayers. Data plotted is the mean±s.d. of 15 individual measurements on duplicate cell cultures.